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Identification of two binding sites for wheat-germ agglutinin on polylactosamine-type oligosaccharides

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The carbohydrate-binding properties of wheat-germ agglutinin (WGA) have been studied by using glycopeptides isolated from the cell surfaces of a cultured murine myeloid cell line (416B). The glycopeptides were passed through affinity columns of lentil lectin (LCA), concanavalin A (Con A) and WGA arranged in series so that material reaching the WGA column had failed to bind to LCA or Con A. WGA-binding glycopeptides were step-eluted with 0.01 M, 0.1 M and 0.5 M-*N*-acetylglucosamine (GlcNAc), to yield weak (WGA-W), intermediate (WGA-I) and strong (WGA-S) affinity fractions. WGA-W and WGA-I contained '*N*'- and '*O*'-linked oligosaccharides bound to separate polypeptides. WGA-S consisted almost entirely of *N*-linked components. Our analytical work was concentrated mainly on the *N*-linked fractions. In these carbohydrates WGA affinity was directly proportional to molecular size but inversely related to *N*-acetylneuraminic acid content. The binding of the weak-affinity fraction was dependent on *N*-acetylneuraminic acid, but the intermediate- and strong-binding species interacted with the lectin by *N*-acetylneuraminic acid-independent mechanisms. *N*-linked glycopeptides in each WGA-binding class were almost totally degraded to monosaccharides by the concerted action of the exoglycosidases neuraminidase, β-galactosidase and β-*N*-acetylglucosaminidase. Treatment with endo-β-galactosidase caused partial depolymerization, yielding some disaccharides but also a heterogeneous population of partially degraded components. These findings suggest that WGA binds with high affinity to internal GlcNAc residues in large oligosaccharides containing repeat sequences of Galβ(1→4)GlcNAcβ(1→3) (i.e. polylactosamine-type glycans). *N*-Acetylneuraminic acid is involved only in low-affinity interactions with WGA. WGA therefore displays an intricate pattern of saccharide specificities that can be profitably utilized for structural analysis of complex carbohydrates.

INTRODUCTION

Wheat-germ agglutinin (WGA) has long been used as a biochemical tool for the investigation of the cellular expression and structure of glycoconjugates (Lotan & Nicolson, 1979; Cummings & Kornfeld, 1982; Irimura & Nicolson, 1984). However, the interactions of WGA with carbohydrates are complex, and many aspects of the sugar-recognition properties of this lectin are poorly understood. WGA binding is strongly inhibited by short sequences of GlcNAc units [GlcNacβ(1→4)GlcNAcβ(1→4)], but GlcNAc itself is a poor inhibitor (Allen *et al.*, 1973; Goldstein *et al.*, 1975). Of the other sugars commonly found in mammalian carbohydrates, only *N*-acetylneuraminic acid shows some inhibitory effect, but it is even weaker than GlcNAc (Bhavanandan & Katlic, 1979). On mammalian cell surfaces, GlcNAc residues have been found in continuous sequences only in *N*-linked oligosaccharides where the trimannosylchitobiose core is covalently attached to asparagine. Interaction of WGA with *N*-linked structures requires a third bisecting GlcNAc together with several short antennae composed of non-reducing GlcNAc termini linked directly to core α-mannose units (Debray *et al.*, 1981; Yamamoto *et al.*, 1981). The binding of such components is weak in the absence of the bisecting GlcNAc residue. These findings are important, since they show that multiple peripheral GlcNAc residues are not good ligands for WGA. Binding of WGA to bisected *N*-linked cores is inhibited by the presence of commonly found complex outer antennae of the structure NeuAc-Gal-GlcNAc (Yamamoto *et al.*, 1981). The question then arises as to what are the saccharide structures that WGA binds to on mammalian cell surfaces.

Several workers have shown that the binding of WGA to cells or isolated glycopeptides is decreased after treatment with neuraminidase (Monsigny *et al.*, 1980; Bhavanandan *et al.*, 1981). *N*-Acetylneuraminic acid residues were therefore implicated as important factors in WGA interactions (Bhavanandan & Katlic, 1979; Monsigny *et al.*, 1980). WGA-binding glycopeptides have been isolated from melanoma cells (Bhavanandan & Katlic, 1979; Bhavanandan *et al.*, 1981) and human mammary-tumour cells (Chandrasekaran & Davidson, 1979). These glycopeptides did not bind to WGA after desialylation, and the WGA reactivity was ascribed to the presence of short sialylated '*O*'-linked oligosaccharides clustered on a central polypeptide chain (Bhavanandan & Katlic, 1979). However, kinetic studies on WGA binding to untreated and neuraminidase-treated cells

Abbreviations used: WGA, wheat-germ agglutinin; LCA, lentil lectin; Con A, concanavalin A; GlcNAc, *N*-acetylglucosamine; RCA_{120}, *Ricinus communis* $agglutinin_{120}$.

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have revealed the presence of *N*-acetylneuraminic acid-independent lectin-reactive sites of very high affinity (Stanley & Carver, 1977; Gallagher *et al.*, 1983).

The present paper reports the isolation of sialylated glycopeptides from the surface membrane of a murine haemopoietic cell line (416B) (Dexter *et al.*, 1979) with WGA. In particulr, the characterization of sialylated *N*-linked poly-*N*-acetyl-lactosamine-type glycopeptides is described. A structural basis for the interaction of WGA with these molecules is proposed.

MATERIALS AND METHODS

Cell cultures

416B cells originated from mouse bone-marrow cultures infected with Friend leukaemia virus (Dexter *et al.*, 1979). They grow in the absence of exogenous growth factors, but are non-tumorigenic (for further details, see Teich *et al.*, 1981). Fresh subcultures were obtained and set up at $(1–3) \times 10^5$ cells/ml in normal growth medium containing either 5 μCi of D-[1-^{3}H]-glucosamine hydrochloride (sp. radioactivity 2–5 Ci/mmol) or 10 μCi of D-[2-^{3}H]mannose (sp. radioactivity 10–20 Ci/mmol) (Amersham International)/ml. These were incubated at 37 °C for 48 h, during which time each culture went through two population doublings.

Isolation of glycopeptides

Washed cell pellets [$(10–20) \times 10^6$ cells] were resuspended in 5 ml of 20 mM-Tris/acetate buffer, pH 7.4, containing 1 mM-calcium acetate, 0.15 M-NaCl and 50 μg of bovine pancreatic trypsin type III (Sigma Chemical Co.)/ml. This was incubated for 15 min at 37 °C, and then neutralized by addition of 250 μg of soya-bean trypsin inhibitor (Sigma). The cells were centrifuged and the supernatant trypsin digest was removed. The trypsin digest was treated with 1.6 ml of *Streptomyces griseus* proteinase (Sigma type VI) at 5 mg/ml in the above buffer for 48 h at 37 °C under toluene. This was followed by a further addition of 0.4 ml of proteinase and incubation for 24 h. The enzyme was destroyed by boiling at 100 °C for 10 min.

Serial affinity chromatography with Sepharose-conjugated LCA, Con A and WGA

Sepharose-conjugated LCA, Con A and WGA were obtained from Pharmacia and contained respectively 2, 10 and 5 mg of lectin/ml of settled gel. Columns (8 cm × 0.65 cm) were connected in series such that the eluate from the LCA column entered the Con A column and the eluate was then passed through the WGA column. Columns were equilibrated with 10 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl. Sample was pumped on to the first column (LCA) and allowed to bind for 15 min. The column series was then washed at 10 ml/h with 14 ml of buffer containing 1 M-NaCl, after which the LCA column was disconnected and the Con A and WGA columns were washed with a further 10 ml of buffer before also being disconnected. Each column was then eluted separately with equilibration buffer followed by different concentrations of specific monosaccharides as described in Fig. 1 (below).

RCA_{120}–agarose chromatography

RCA_{120}, coupled to an agarose gel was purchased from Vector Laboratories, Burlingame, CA, U.S.A., and contained 4 mg of RCA_{120}/ml of settled gel. A column (8 cm × 0.65 cm) was run as described above and eluted with 0.01 M- and 0.1 M-galactose.

Ion-exchange chromatography

Anion exchange was carried out on a column (7 cm × 0.65 cm) of Whatman DEAE-cellulose (DE-52) equilibrated with 10 mM-Tris/acetate buffer, pH 7.4. Samples in equilibration buffer were pumped on to the column and then eluted at 15 ml/h with a linear 0–0.2 M-NaCl gradient, followed by a steeper, second gradient from 0.2 M to 1 M-NaCl. Full details are given in the legend to Fig. 5 (below).

Alkaline elimination

This was performed as described by Mayo & Carlson (1970). Samples were incubated with 0.1 M-NaOH in 1 M-$NaBH_4$ for 48 h at 37 °C under an N_2 atmosphere. Samples were then cooled to 4 °C and neutralized by dropwise addition of 1 M-acetic acid, with Phenol Red as indicator.

Acid hydrolysis

For sialic acid removal, freeze-dried samples were dissolved in 0.1 M-HCl and incubated at 80 °C for 1 h. The hydrolysate was neutralized by dropwise addition of 0.1 M-NaOH, with Phenol Red as indicator.

Glycosidase treatments

Neuraminidase. Freeze-dried material was dissolved in 0.1–0.2 ml of 50 mM-citrate buffer, pH 5.0, containing 0.1 unit of *Clostridium perfringens* neuraminidase (Sigma) and incubated at 37 °C for 18 h.

Endo-β-galactosidase. Enzyme from *Escherichia freundii* was kindly donated by Dr. Minoru and Dr. Michiko Fukuda of La Jolla Cancer Research Foundation, La Jolla, CA, U.S.A. (Fukuda, 1981; Scudder *et al.*, 1984). Samples were incubated at 37 °C overnight with 0.135 unit of enzyme/ml in sodium acetate buffer, pH 5.8. These were then freeze-dried and reconstituted with buffer containing 150 munits of enzyme/ml to give a final enzyme concentration of 0.52 unit/ml and incubated for a further 40 h at 37 °C.

Exoglycosidases. Freeze-dried material was dissolved with 100 μl of 10 mM-phosphate/citrate buffer, pH 5.0, containing, 0.02% NaN_3, 0.1–0.3 unit of β-galactosidase (from jack bean; Sigma type VII) and 1.3–2.0 unit of β-*N*-acetylglucosaminidase (from jack bean; Sigma). This was incubated at 37 °C for 40 h, followed by the further addition of 50 μl of each enzyme (0.04 and 0.33 unit), and further 7 h incubation. On some occasions neuraminidase (0.1 unit) was added to the incubation mixture.

Paper chromatography

[^{3}H]GlcNAc-labelled samples. Desalted samples and reference sugar were hydrolysed for 7 h with 2 M-HCl in sealed tubes at 100 °C. The acid was removed by repeated evaporation over P_2O_5 and NaOH *in vacuo*. Descending chromatography was performed on a 10 cm-wide × 50 cm-long sheet of Whatman no. 1 paper, with solvent system 1 (n-butanol/pyridine/water, 6:4:3, by vol.) or 2 (ethyl acetate/pyridine/water, 12:5:4, by vol.). Chromatograms

were dried, and tracks for radioactivity counting were cut into 0.5 cm strips. Remaining tracks were stained with alkaline silver oxide (Trevelyan *et al.*, 1950) or a 2% (w/v) solution of ninhydrin (Sigma) in acetone.

[^{3}H]Mannose-labelled glycopeptides. These were hydrolysed with 0.25 M-H_2SO_4 at 80 °C for 5 h and then deionized on Amberlite MB-3 mixed-bed ion-exchange resin (Sigma). Neutral sugars were chromatographed as described above.

Reference oligosaccharides. (1) A mucin-type tetrasaccharide:

NeuAcα(2→3)Galβ(1→3)GalNAc-[^{3}H]ol
2–6 ↑
NeuAc

purified from fetuin glycopeptide was kindly provided by Dr. A. Corfield, Department of Medicine, University of Bristol, Bristol, U.K.

(2) Disaccharide was prepared by treatment of mucin-type tetrasaccharide (1) with neuraminidase.

(3) α_1-Acid glycopeptides, a ^{14}C-labelled mixture of di-, tri- and tetra-antennary complex sialoglycopeptides, were kindly donated by Dr. H. Debray, Laboratoire de Chimie Biologique, University of Lille, Lille, France.

RESULTS

Affinity chromatography

Pronase-digested, trypsin-sensitive glycopeptides from the surfaces of 416B cells metabolically labelled with either [^{3}H]glucosamine or [^{3}H]mannose were run on the LCA, Con A and WGA affinity-column series (Fig. 1). The profiles for the [^{3}H]glucosamine-labelled extracts were characterized by large amounts of weak-affinity LCA-binding material, eluted with 5 mM-α-methyl mannoside (LCA-W), and three substantial WGA-binding peaks which exhibited weak (WGA-W), intermediate (WGA-I) and strong (WGA-S) affinity for the lectin on the basis of their elution with 0.01 M-, 0.1 M- and 0.5 M-GlcNAc respectively. Despite the high level of [^{3}H]glucosamine label in these fractions, they each contained considerably less label, when compared with the LCA- and Con A-binding glycopeptides, when [^{3}H]mannose was used as the radio labelled precursor (cf. Figs. 1*a* and 1*b*). The non-binding fraction, i.e. that which passes through all three columns without binding, consists of only about 20% of the total labelled glycopeptides when the contribution of free label is subtracted. The lectin affinity-column series therefore represents an effective means for fractionating cell-surface glycopeptides. The carbohydrate-binding specificities of LCA (Kornfeld *et al.*, 1981) and Con A (Narasimhan *et al.*, 1978; Baenziger & Fiete, 1979*a*) are reasonably well established. With the exception of a small amount of glycopeptides in LCA-S, none of the Con A- and LCA-binding fractions interacted with WGA. This suggests that most low-M_r bi- and tri-antennary complex glycans are not recognized by WGA (see below). Notably a standard preparation of glycopeptides from α_1-acid glycoprotein also failed to bind to WGA.

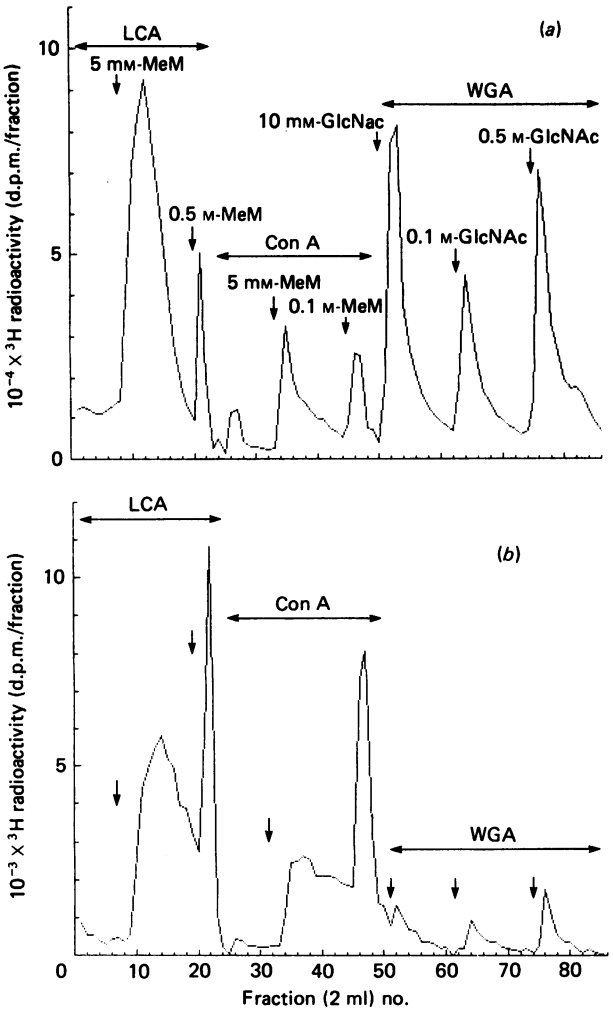

Fig. 1. Serial lectin affinity chromatography of cell-surface glycopeptides labelled with either [^{3}H]glucosamine (*a*) or [^{3}H]mannose (*b*)

Pronase-digested glycopeptides were applied to the lectin affinity columns equilibrated with 10 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl. After the sample (1–2 ml) was run into the first column (LCA), 14 ml of 10 mM-Tris/HCl, pH 7.4, containing 1 M-NaCl, was pumped through the columns to elute material that may bind to the lectins by ionic interactions. The LCA column was then removed from the series and Con A and WGA columns eluted with a further 10 ml of high-salt buffer. All three columns were then separated from each other and LCA and Con A columns were eluted with a further 14 ml of high-salt buffer. The LCA and Con A columns were then eluted first with 34 ml of 5 mM-α-methyl D-mannoside (MeM), then with 22 ml of 0.5 M-α-methyl D-mannoside. The WGA columns were step-eluted with 24 ml each of 10 mM-, 0.1 M and 0.5 M-GlcNac. Arrows on the graphs indicate the points at which each step elution was begun. All sugar solutions were made up in equilibration buffer. Fractions (2 ml) were collected at a flow rate of 8–10 ml/h.

Molecular size of WGA-binding glycopeptides: influence of reductive alkaline elimination

Molecular-size comparisons performed on Sephadex G-100 showed that WGA-binding glycopeptides were distinct from those of LCA and α_1-acid glycopeptides in

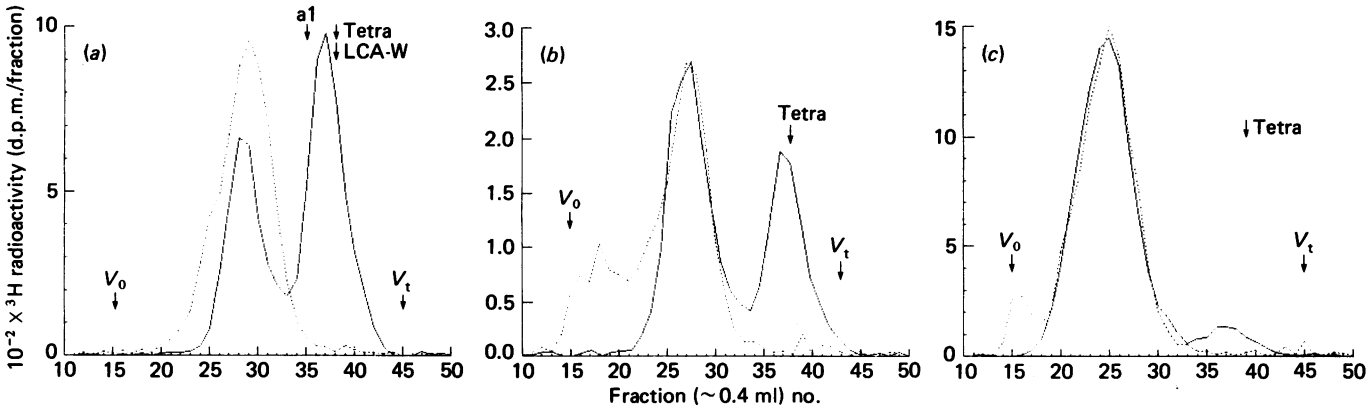

Fig. 2. Effect of alkaline-borohydride treatment on the molecular size of WGA-binding glycopeptides

WGA-binding glycopeptides, metabolically labelled with [^{3}H]glucosamine, were applied to a Sephadex G-100 column (0.65 cm × 48 cm) before and after treatment with alkaline borohydride (see the Materials and methods section) and eluted at about 3 ml/h with 0.25 M-NH_4HCO_3. Each profile represents a separate column run. (*a*) WGA-W; (*b*) WGA-I; (*c*) WGA-S; - - - -, untreated samples; ——, alkaline-borohydride-treated samples; V_0, void volume (Dextran Blue); V_t, total included volume (sodium dichromate). The peak elution volumes of reference mucin-type tetrasaccharide (Tetra), α_1-acid glycopeptides (a1) and LCA-W glycopeptides (LCA-W) are indicated.

being of much higher M_r (Fig. 2). WGA affinity appears to be related to molecular size, WGA-S being the largest species and WGA-W the smallest.

Treatment with alkaline borohydride (0.1 M-NaOH/1 M-$NaBH_4$) had a most interesting effect on the G-100 profiles (Fig. 2). Around 50% of the ^{3}H label in WGA-W and WGA-I appeared as low-M_r products, whereas the rest of the material continued to give $K_{av.}$ values at peak maxima that were identical with those shown by the untreated preparations. This finding suggests that the alkali-sensitive and alkali-resistant fractions (presumably consisting of *O*- and *N*-linked glycans respectively) were on separate polypeptides. Very little radioactivity (< 10%) was released by alkali from the WGA-S glycopeptides (Fig. 2*c*).

Chromatography on Bio-Gel P-6 (Fig. 3) showed that the alkali-eliminated components were eluted in a similar position to the *O*-linked tetrasaccharide standard, the structure of which was displayed in the Materials and methods section. The alkali-released tetrasaccharides did not bind to WGA. When treated with neuraminidase, they yielded free sialic acid and a disaccharide, as judged by rechromatography on Bio-Gel P-6.

The amino sugars of alkali-sensitive and -resistant materials were determined by acid hydrolysis and paper chromatography. The sensitive component contained mostly galactosamine (80%), probably originating from the protein-linkage region, together with smaller amounts of glucosamine. By contrast, the alkali-resistant fraction contained only glucosamine. These findings support an interpretation that the alkali-resistant material is composed of *N*-linked oligosaccharides that always contain glucosamine but rarely galactosamine. When [^{3}H]mannose-labelled glycopeptides were subjected to alkali treatment, no low-M_r radiolabelled oligosaccharides were released. In mammalian carbohydrates, mannose is found only in *N*-linked carbohydrates. Acid hydrolysis and paper chromatography showed that about 30% of the [^{3}H]mannose label had been converted to [^{3}H]fucose in the presumed *N*-linked glycopeptides, but neither of these two neutral sugars appeared in the WGA-binding *O*-linked glycans (paper-chromatography results not shown).

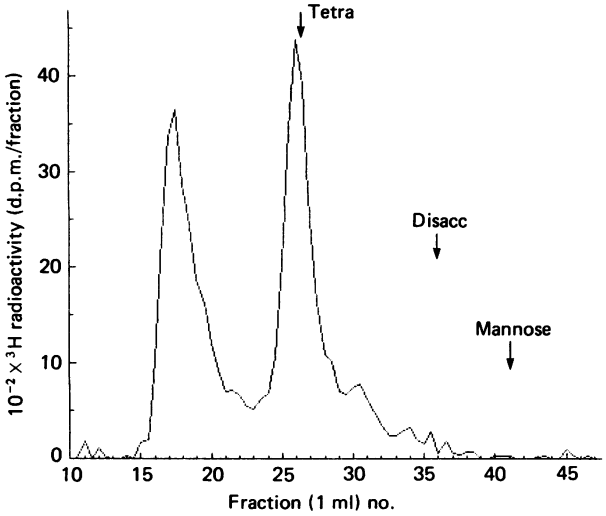

Fig. 3. Gel filtration on Bio-Gel P-6 of alkaline-borohydride-treated WGA-I glycopeptides

A [^{3}H]glucosamine-labelled sample of WGA-I was treated with alkaline borohydride and applied to a Bio-Gel P-6 column (1.2 cm × 57 cm) and eluted at about 5 ml/h with 0.25 M-NH_4HCO_3. Elution volumes of reference mucin-type tetrasaccharide (Tetra), disaccharide (Disacc; neuraminidase-treated Tetra) and [^{14}C]mannose are indicated. Alkali-stable material, i.e. *N*-linked chains (eluted at about 15–19 ml), and alkali-labile *O*-linked fractions (25–29 ml) were separately pooled for further analysis.

Ion-exchange chromatography

To confirm that the *N*- and *O*-linked oligosaccharides were bound to separate polypeptides, a pooled preparation of WGA-binding materials (WGA-W+WGA-I+WGA-S) was fractionated by DEAE-cellulose chromatography (Fig. 4) by using an NaCl gradient as eluent. Two

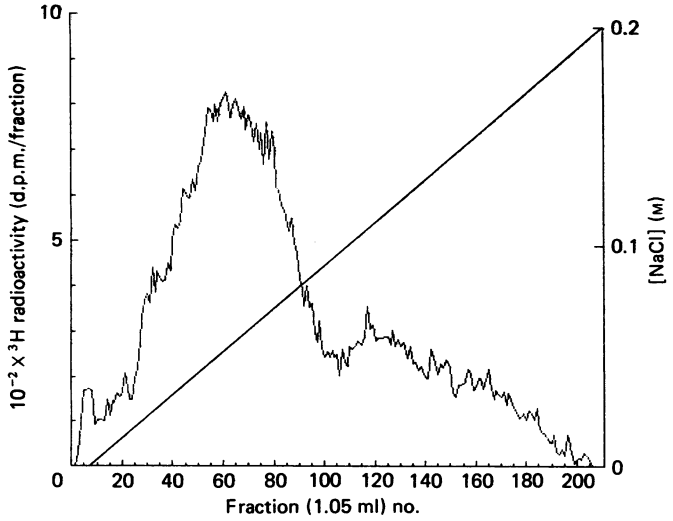

Fig. 4. Ion-exchange chromatography of [^{3}H]glucosamine-labelled WGA-binding glycopeptides

This was performed on a column (0.65 cm × 7 cm) of DEAE-cellulose anion-exchange gel equilibrated to pH 7.4 with 10 mM-Tris/acetate buffer. A pooled sample of WGA-binding glycopeptides (WGA-W + WGA-I + WGA-S) each at their unfractionated proportions was pumped on to the column at 15 ml/h and then eluted with a two-stage linear gradient of NaCl (0.0→0.2 M; 0.2 M→1 M) (see Materials and methods section). Only the 0→0.2 M section is shown (diagonal 'straight' line) since no radioactivity eluted in the remainder. Material eluted between 0.0 and 0.1 M- and between 0.1 M and 0.2 M-NaCl was separately pooled for further analysis.

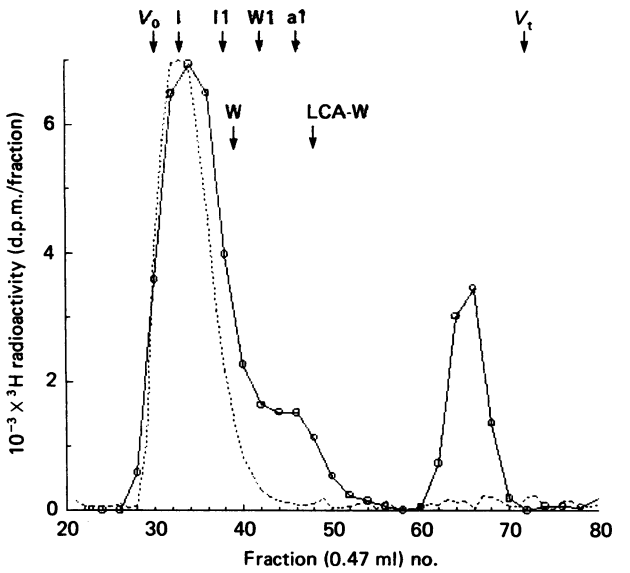

Fig. 5. Effect of neuraminidase treatment on WGA-binding glycopeptides

Alkali-stable glycopeptides from each WGA-binding population were treated with neuraminidase and applied to a Sephadex G-50 column (0.9 cm × 60 cm), which was eluted with 0.25 M-NH_4HCO_3 at about 6 ml/h. The Figure shows results for the WGA-S fraction. Also shown are the peak elution positions of untreated WGA-W and WGA-I ('W' and 'I' respectively on the graph) and their neuraminidase-treated counterparts (W1 and I1 respectively on the graph). a1 and LCA-W mark the elutions of α_1-acid glycopeptides and low-affinity LCA-binding material. ----, Control sample; ——, neuraminidase-treated sample.

major peaks were observed to be eluted at 0→0.09 M and 0.1→0.2 M-NaCl. These were separately pooled and treated with alkali. The low-affinity glycopeptides were predominantly alkali-stable, and the high-affinity DEAE-binding peak was almost all alkali-labile. This indicates that the *N*- and *O*-linked chains are indeed found on different polypeptides. The strong polyanionic properties of the *O*-linked materials are due to a higher sialic acid content (about 50% of ^{3}H label in sialic acid compared with 20% on the *N*-linked chains; see below). None of the glycopeptides was labelled when $Na_2{}^{35}SO_4$ was used as the radioisotopically labelled precursor.

Characterization of *N*-linked WGA-binding glycopeptides

N-Linked chains from each WGA-binding fraction were isolated by treatment with alkali and gel filtration as described above, and then subjected to the following analyses.

***N*-Acetylneuraminic acid content.** *N*-Linked glycopeptides were treated with neuraminidase and then run on Sephadex G-50 (Fig. 5). Efficient *N*-acetylneuraminic acid removal was demonstrated by using mild acid treatment, which gave results identical with those produced by neuraminidase. The WGA-binding glycopeptides contain a relatively high proportion of ^{3}H label as *N*-acetylneuraminic acid, but WGA affinity is inversely proportional to *N*-acetylneuraminic acid content. The percentages of ^{3}H label released from each glycopeptide class were 27.5% for WGA-W, 25% for WGA-I and 19% for WGA-S. Fig. 5 shows the effect of *N*-acetylneuraminic acid removal on WGA-S: the desialylated components were eluted near the excluded volume, indicating that they are still large molecules. Desialylation also had only a small effect on the molecular sizes of WGA-W and WGA-I (Fig. 5).

Affinity chromatography with RCA_{120}. Intact glycopeptides of WGA-W and WGA-I did not bind to RCA_{120}, but desialylated counterparts all bound to the lectin (results not shown). This suggests a terminal sugar sequence of NeuAc-Gal in which most of the galactose residues are substituted with *N*-acetylneuraminic acid. However, 60% of WGA-S binds to RCA_{120} without neuraminidase treatment, demonstrating that some of these glycopeptides contain terminal RCA_{120}-interacting galactose. This finding is consistent with the lower sialic acid content of this fraction.

It should be noted that RCA_{120} can bind, albeit with lower affinity, to subterminal galactose residues substituted by *N*-acetylneuraminic acid linked $\alpha(2\rightarrow6)$ rather than $\alpha(2\rightarrow3)$ (Baenziger & Fiete, 1979*b*). However exoglycosidase data (see below) support the view that only WGA-S has exposed galactose termini.

WGA affinity of desialylated *N*-linked glycopeptides. *N*-Linked glycopeptides separated from the *O*-linked glycans by alkali elimination continued to reproduce their WGA-binding characteristics. Glycopeptides from each WGA binding class were desialylated and re-tested for WGA affinity. Those from WGA-W showed no

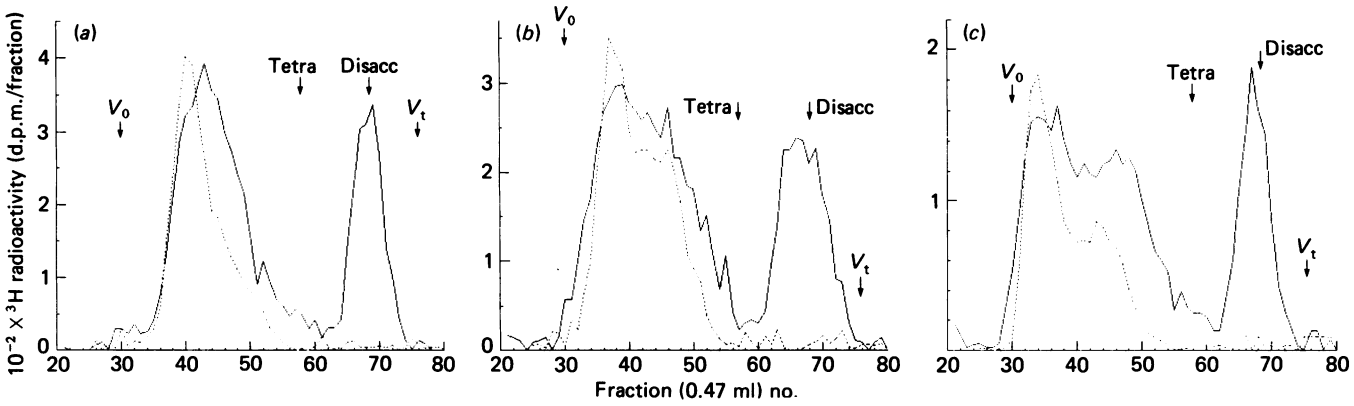

Fig. 6. Endo-β-galactosidase treatment of WGA-binding glycopeptides

[^{3}H]Glucosamine-labelled *N*-linked glycopeptides were treated with endo-β-galactosidase and applied to a Sephadex G-50 column used as described in the legend to Fig. 5. The elution position of standard mucin-type tetrasaccharides (see the Materials and methods section for details) and disaccharides produced by neuraminidase treatment of these tetrasaccharides are indicated. (*a*) WGA-W; (*b*) WGA-I; (*c*) WGA-S. - - - -, Control samples; ——, endo-β-galactosidase-treated samples.

interaction with WGA after removal of *N*-acetylneuraminic acid. However WGA-S continued to bind with characteristic strong affinity (eluted with 0.5 M-GlcNAc). WGA-I also showed *N*-acetylneuraminic acid-independent binding to WGA, but this time the affinity of the preparation was *increased*, requiring 0.5 M-GlcNAc for elution instead of 0.1 M (results not shown). These findings suggest that WGA is probably binding to internal GlcNAc residues in the intermediate- and strong-affinity fractions.

Endo-β-galactosidase treatment. This enzyme specifically degrades repeat *N*-acetyl-lactosamine sequences at galactose residues:

GlcNAcβ(1→3)Galβ(1→)4GlcNAcβ(1→)3
(Fukuda, 1981)

N-Linked WGA-binding glycopeptides were treated with endo-β-galactosidase and run on Sephadex G-50 (Fig. 6). The amount of radioactivity released as a low-M_r peak, approximately equivalent in size to disaccharides, was about 30% in each case. A range of larger oligosaccharides was also detected. Some polylactosamine-type sequences must therefore be present in each of our glycopeptide classes. Incomplete degradation could be due to branching at potentially susceptible galactose units (Fukuda *et al.*, 1984).

Exoglycosidase treatment. To confirm the presence and full extent of repeat Gal-GlcNAc sequences, *N*-linked chains of pooled WGA-W and WGA-I were treated by the concerted action of neuraminidase, β-galactosidase and β-*N*-acetylglucosaminidase. The near-complete degradation to low-M_r sugars, which were eluted near the total volume on Sephadex G-50 (Fig. 7), gave a clear indication that the WGA-binding glycopeptides contain extended Gal-GlcNAc sequences. A small amount of larger heterogeneous material was also eluted between fractions 45 and 60. Some of this radioactivity may represent core-region ^{3}H label, which would be resistant to glycosidase attack. Without neuraminidase treatment, β-galactosidase and β-*N*-acetylglucosaminidase caused no degradation of WGA-W and WGA-I.

WGA-S was also extensively degraded by the concerted action of neuraminidase, galactosidase and

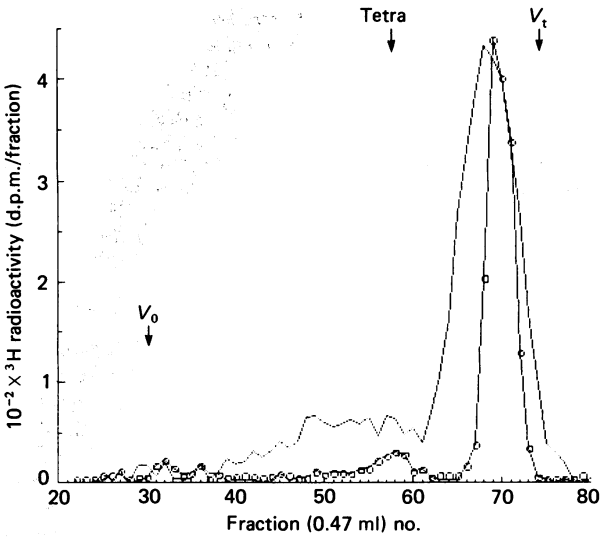

Fig. 7. Exoglycosidase treatment of WGA-binding glycopeptides

N-Linked WGA-binding glycopeptides, labelled with [^{3}H]glucosamine, were treated simultaneously with neuraminidase, β-galactosidase and β-*N*-acetylglucosaminidase and fractionated on Sephadex G-50 as described in Fig. 5. ——, Pooled WGA-W and WGA-I; □—□, WGA-S. 'Tetra' marks the elution of mucin-type tetrasaccharide.

glucosaminidase (Fig. 7), but some degradation (15%; results not shown) of this material was achieved by the latter two enzymes in the absence of neuraminidase. This is indicative of some galactose termini and is consistent with the RCA-binding properties of a portion of the WGA-S fraction.

DISCUSSION

WGA expresses a complex series of interactions with mammalian oligosaccharides. This is well illustrated by the existence of five complementation groups of WGA-resistant cell lines, each with different lesions in carbohydrate synthesis (Stanley, 1980). The structural changes arising from these lesions are not fully resolved,

but one class of resistant cells produces saccharides enriched in fucose at the expense of sialic acid (Finne *et al.*, 1980), whereas another is unable to synthesize large, polylactosamine-type structures, and the prematurely truncated glycans lack normal levels of sialic acid (Dennis *et al.*, 1984). In both instances it was assumed that the loss of *N*-acetylneuraminic acid was the primary reason for the WGA-resistance.

Our findings emphasize the diverse nature of the carbohydrates that show complementarity with WGA. Essentially two mechanisms of lectin binding have been identified: high-affinity, *N*-acetylneuraminic acid-independent binding and low-affinity sialic acid-dependent interactions. High-affinity binding occurs with polylactosamine-type oligosaccharides in which the complementary sugars are most probably internal GlcNAc residues arranged in specific linear and/or branched sequences. There appears to be an M_r-dependence for strong interactions with WGA (cf. molecular sizes of WGA-S and WGA-W; Figs. 2 and 5). By demonstrating strong reactivity with internal sugars in complex carbohydrates, WGA has properties consistent with a Class IIb or heterotypic endolectin (Gallagher, 1984). However, it will be recalled that WGA also binds strongly to sequences of identical sugar units as found, for example in tri-*N*-acetylchitotriose (see the Introduction), and this property is typical of Class IIa, or homotypic, endolectins. The eventual classification of WGA must await studies showing whether sequences of identical or non-identical sugars form the highest-affinity ligands.

Low-affinity interactions with *N*-linked and *O*-linked oligosaccharides found in WGA-W requires external *N*-acetylneuraminic acid residues, which are probably clustered in the '*O*'-linked species (Fig. 3). These latter materials are resistant to Pronase, suggesting that they are in close proximity along a central polypeptide. We have not determined the number of *N*-acetylneuraminic acid residues in each molecule of the *N*-linked oligosaccharides found in WGA-W. However, fully sialylated tetra-antennate glycans with single *N*-acetyl-lactosamine units in each antenna fail to bind to WGA (Debray *et al.*, 1981). It seems unlikely that our WGA-W oligosaccharides have more than four antennae. The WGA-binding site in these weak-interacting species could involve both external *N*-acetylneuraminic acid and internal GlcNAc residues in sequences of NeuAc-Gal-GlcNAc-Gal-GlcNAc.... It may be significant that these low-affinity structures are of smaller molecular size than are WGA-I or WGA-S (Figs. 2 and 5). They may therefore lack the appropriate number and arrangement of internal GlcNAc units for high-affinity WGA binding and require *N*-acetylneuraminic acid to complete the formation of a reactive sequence. NeuAc has a much lower affinity for WGA than has GlcNAc (itself a rather poor inhibitor of lectin binding; see Bhavanandan & Katlic, 1979; Allen *et al.*, 1973), which may account for the correspondingly low affinity of oligosaccharides which bind to WGA by an *N*-acetylneuraminic acid-dependent mechanism.

It was rather striking that WGA-I was converted into a stronger-binding species after removal of *N*-acetylneuraminic acid. However, sialylation was incomplete in the naturally occurring WGA-S fraction. *N*-Acetylneuraminic acid may impede access of WGA to the highest-affinity domains of polylactosamine sequences.

This is not the first study to demonstrate that polylactosamine glycans can bind to WGA (Carlsson *et al.*, 1976; Krusius *et al.*, 1978; Eckardt & Goldstein, 1983). What is new in the present work is the clear demonstration that internal GlcNAc residues, not external *N*-acetylneuraminic acids, form the highest-affinity binding sites, that *N*-acetylneuraminic acid itself has opposing influences on the carbohydrate recognition process and that strong binding to the native oligosaccharides seems to require a minimum size and perhaps also a specific branched arrangement of polylactosamine sequences. Lack of reactivity of the relatively low-M_r desialylated WGA-W, which clearly contains Gal-β(1→4)GlcNac repeat sequences (Fig. 6), provides the evidence that some specialization in structure is necessary for WGA binding. It should be interesting to examine the reactivity of these oligosaccharides with 'I' and 'i' monoclonal antibodies which bind specifically to branched and linear polylactosamine-type structures (Feizi, 1984).

Serial lectin affinity chromatography using LCA, Con A and WGA provides valuable information on the carbohydrate structures of most (about 80%) of trypsin-extracted glycopeptides from 416B cells (Fig. 1). The high content of low-affinity LCA-binding material indicates a considerable enrichment of fucosylated, biantennate (and possibly also triantennate) *N*-linked glycans (Kornfeld *et al.*, 1981), whereas the relatively small amount of glycopeptides binding with weak affinity to Con A is indicative of a low-frequency non-fucosylated biantennary components (Krusius *et al.*, 1978; Baenziger & Fiete, 1979*a*). The strong-binding Con A fraction probably contains mainly high-mannose oligosaccharides, since [^{3}H]mannose gave better labelling than [^{3}H]glucosamine (Fig. 1) and this fraction did not contain any *N*-acetylneuraminic acid. As we have demonstrated here, the abundance of [^{3}H]glucosamine-labelled, WGA-reactive glycopeptides of different lectin affinities is due to *N*-linked polylactosamine constituents of variable size and perhaps with distinctive branching patterns, together with glycopeptides containing short, sialylated *O*-linked oligosaccharides (Figs. 2 and 3). The presence of these '*O*'-linked materials, along with the repeat Gal-GlcNAc sequences in the *N*-linked fraction, explains the low labelling of WGA-binding glycopeptides with [^{3}H]-mannose when compared with the relatively abundant labelling with [^{3}H]glucosamine (cf. Figs. 1*a* and 1*b*). Further elucidation of the precise structural requirements for carbohydrate binding to WGA will make this lectin a particularly powerful reagent for carbohydrate sequence analysis.

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